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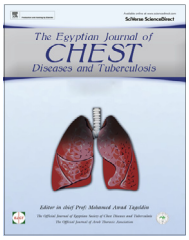

ORIGINAL ARTICLE

Association between environmental tobacco smoke exposure and lung cancer susceptibility: Modification by antioxidant enzymes genetic polymorphisms

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KEYWORDS

Environmental tobacco smoke;
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Abstract *Background:* Environmental tobacco smoke (ETS) is the primary etiological factor of lung cancer. However, only 10–15% of smokers develop lung cancer, suggesting genetic role in modifying individual susceptibility to lung cancer. Antioxidant enzyme functional genetic polymorphisms should be considered.

Aim of the work: The present study aimed to evaluate the role of antioxidant enzyme activity and genetic polymorphisms in modifying the susceptibility to lung cancer among individuals exposed to ETS.

Subjects and methods: A total of 150 male subjects were divided into three groups: 50 lung cancer patients, 50 chronic smokers and 50 passive smokers. Genotyping of mEH exon 3 ($Tyr^{113}Hist$) and exon 4 ($Hist^{139}Arg$) polymorphisms was done by PCR–RFLP technique. MnSOD ($Val^{16}Ala$) polymorphism was detected by Real time-TaqMan assay. Erythrocyte MnSOD activity was measured spectrophotometrically.

Results: ETS exposed individuals (both active and passive smokers) who carried His allele of mEH exon3 have 2.9-folds increased risk of lung cancer (***OR* 2.9 *P* < 0.001**). Also ETS exposed carriers of Arg allele of mEH exon 4 have 2.1-folds higher risk to lung cancer **(*OR* 2.1 *P* = 0.024)**. However no association between MnSOD $Val^{16}Ala$ polymorphism and lung cancer was detected among ETS **(*OR* 1.6 *P* = 0.147),** although lung cancer group had significantly lower MnSOD activity than chronic or passive smokers groups **(*P* = 0.03)**.

Conclusion: Exons 3 and 4 polymorphisms of the mEH gene may contribute to lung cancer susceptibility through disturbed antioxidant balance. However, this was not the case with MnSOD $Val^{16}Ala$ SNP. Antioxidant enzymes may modulate the influence of ETS exposure on lung cancer risk.

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Introduction

Environmental tobacco smoke (ETS) refers to the exposure of a non-smoker to the smoke produced from cigarettes consumed by another person. It is also called second-hand smoke, passive smoking or involuntary smoking [1]. Exposure to tobacco smoke, either by active or passive smoking, is the primary etiologic factor responsible for lung cancer. Long-term tobacco smoke exposure was found to cause 80–90% of lung cancers worldwide [2]. Although most of lung cancer patients are smokers or ex-smokers, in fact many of them are also non-smokers and only 10–15% of smokers may develop lung cancer in their lifetime, suggesting that environmental factors (mainly tobacco smoke) interact with multiple polymorphic genes to influence cancer susceptibility [3,4].

Mild oxidative stress occurs on a daily basis and is a key factor in maintaining homeostasis. However, strong, acute, or chronic oxidative stress disrupts this delicate homeostasis and causes oxidative damage to lipids, proteins and nucleic acid molecules leading to increased vulnerability to malignant diseases [5].

Microsomal epoxide hydrolase (mEH), a phase II metabolic enzyme, catalyzes the hydrolysis of epoxides from polycyclic aromatic hydrocarbons and aromatic amines of cigarette smoke [6]. Although this hydrolysis is generally a detoxification reaction as less reactive and more water soluble dihydrodioles are produced, in case of some hydrocarbons such as benzo(α)pyrene, present in tobacco smoke, more highly reactive and mutagenic compounds are generated. Thus mEH exhibits a dual role of procarcinogen detoxification and activation depending on the substrate [7,8]. In the coding region of mEH gene, two common polymorphisms are characterized within exons 3 and 4. In exon 3, a T > C transition resulting in Tyr113His substitution, is associated with 40-50% decrease in the in vitro activity of mEH, and thus this allelic conversion has been referred to as the "slow" allele. The second variant is characterized by an A > G transition in exon 4 causing His139Arg substitution, and is associated with 25% increase of enzyme activity. This allele has been called the "fast" allele. The distance between exon 3 and exon 4 is 6696 base pairs [9].

Given the known differential effect of mEH alleles in the detoxification of procarcinogens, it has been proposed that these polymorphisms may affect cancer risk [10].

A number of antioxidants enzymes are involved in the scavenging of reactive oxygen species (ROS), including the superoxide dismutase (SOD) family members (Mn, Cu and ZnSOD). Theses enzymes catalyzes the dismutation of superoxide anion ($O_2^{-\bullet}$) to form hydrogen peroxide (H_2O_2), which is further detoxified to water by glutathione peroxidase [11]. MnSOD is the only SOD essential for life, and the major antioxidant in the mitochondria. MnSOD precursor protein is synthesized with a cleavable N-terminal mitochondrial targeting sequence (MTS) which derives the mitochondrial import of MnSOD from the cytoplasm. Genetic polymorphism at codon 16 of MnSOD/MTS leads to substitution of alanine (GCT) for valine (GTT) T > C (Val 16 Ala, rs4880) [12]. This polymorphism was reported to be functional in affecting the transport of the enzyme into mitochondria with the Ala variant accounting for more efficient importation [13]. A number of molecular studies have been conducted to examine the link between MnSOD Val 16 Ala and cancer susceptibility [14–16], but the results remain inconsistent. The aim of the present study is to evaluate the role of mEH and MnSOD enzyme activity and genetic polymorphisms in modifying the susceptibility to lung cancer among individuals exposed to ETS.

Patients and methods

This cross-sectional study was performed in the Chemical Pathology Department in collaboration with the Chest Diseases Department, Kasr Al Aini Faculty of Medicine, Cairo University, from June 2011 until March 2013.

The study was conducted on 150 male subjects with their age ranged between 35 and 70 years, divided into three groups each of 50 subjects as follows: **Group I**: Lung cancer patients diagnosed clinically, radiologically and confirmed by histopathological examination of bronchoscopic or CT guided biopsy. **Group II:** Chronic smokers. They have been smoking for at least 10 years with a minimum of 1 pack/day i.e. (smoking index with a minimum of 10 pack/year). **Group III**: Healthy passive smokers, with no medical history of lung disease (no cough, expectoration or shortness of breath). All subjects were asked about their age, chest symptoms, other co-morbidities, smoking history to calculate smoking index (by multiplying the number of cigarette packs smoked/day by the number of years the person has smoked (pack/year) according to the National Cancer Institute (USA) definition of pack/year [17].

Exclusion criteria

Subjects suffering from other co-morbidities which may lead to oxidative stress such as diabetes, cardiac disorders, severe infections, severe liver and kidney disease are excluded.

Specimen collection and storage

All subjects in this study were informed and verbal consents were taken. Six ml venous blood was withdrawn from all subjects and divided into 3 parts: (a) Two ml was collected in a sterile EDTA vacutainer for DNA extraction. Samples were kept frozen at −20 °C till the time of analysis. (b) Two ml was collected in EDTA containing tube for measurement of erythrocyte MnSOD activity. (c) Two ml was collected on plain tubes, left for 10 min to clot and then centrifuged at 3000 rpm for 5 min, to separate serum for routine laboratory investigations (liver and kidney functions) for the exclusion criteria.

DNA preparation

Extraction of genomic DNA from sterile EDTA anticoagulated blood samples was done using QIAamp DNA blood Mini kit (Qiagen, Hilden, Germany) by silica-gel spin columns [18].

Analysis of mEH gene polymorphisms (exon 3 and exon 4) using Polymerase chain reaction (PCR) followed by Restriction Fragment Length Polymorphism (RFLP) analysis

The PCR-RFLP of extracted genomic DNA was performed as described by Cheng et al. [18]. DNA amplification was performed in Gradient thermal cycler (Professional thermocycler,

Biometra, Applied Biosystem, California, USA), after adjusting the thermal profile to initial denaturation at 95 °C for 10 min followed by 35 cycles of 30 s at 94 °C, annealing temperature of 55 °C (exon 3) or 62 °C (exon4) for 30 s and extension at 72 °C for 30 s. Specific Oligonucleotide primers were synthesized by Fermentas (Fermentas UAB, V. Graiciuno 8, Lithuania) as follows: exon 3 sense: 5′-GATCGATAAGTTCCGTTTCACC-3; Anti-sense 5′-ATCCTTAGTCTTGAAG TGAGGAT-3′ (engineered base change, G to A, underlined); exon 4 sense: 5′-ACATCCACTTCATCC ACGT-3′; Anti-sense: 5′-ATGCCTCTGAGAAGCCAT-3′. Each PCR product was digested with Eco RV (exon 3) or Rsa I (exon 4) (Fermentas, Thermo Fisher scientific, USA), separated by electrophoresis on 3% agarose gel, stained with ethidium bromide and transilluminated with ultraviolet light. The exon 3 wild-type allele was expected to yield 140- and 22-base-pair (bp) fragments, whereas the variant allele remained an uncleaved 162-bp fragment (Fig. 1). Conversely, the exon 4 wild-type allele remained an uncleaved 210-bp fragment, whereas the variant allele was expected to yield 164- and 46-bp fragments (Fig. 2). According to the report of **Smith and Harrison** [19], the four groups of putative mEH activity phenotypes were classified as follows: normal (no mutation or heterozygous for both exon 3 and 4), fast (at least one fast exon 4 allele and no exon 3 mutations), slow (one slow exon 3 allele), and very slow (two exon 3 slow alleles).

Analysis of MnSOD (Val 16 Ala) polymorphism by Real time PCR technique using TaqMan SNP genotyping assay [20]

Real-time PCR allelic discrimination was designed using TaqMan SNP Genotyping Assays (Applied Biosystems) and performed on Step One™ Real Time PCR System (Applied Biosystems, Foster City, CA), using the fluorogenic 5′nuclease with TaqMan minor groove binder (MGB) probes to define the MnSOD gene SNP c.47T > C, Val16Ala (rs 4880), assay ID: C_8709053_10. The wild type TaqMan MGB probe was VIC labeled (Allele 1) and the mutant probe was FAM labeled (Allele 2).

The final volume of each reaction was 25ul, consisting of 12.5 ul TaqMan Universal PCR Master Mix (2X) which contained AmpliTaq-Gold DNA polymerase, 1.25ul assay mix (20X) contained primers and probes, 5ul genomic DNA, and 6.25ul nuclease free water. Negative control (no DNA template) was run to ensure that there was no amplification of contaminating DNA. The amplification reactions were carried out with initial hold step at 95 °C for 10 min for activation of AmpliTaq-Gold DNA polymerase followed by 40 cycles of three-step PCR: denaturation at 92 °C for 15 s, annealing at 60 °C for 30 s and extension at 60 °C for 30 s. The fluorescence signal increases when the probe with the exact sequence match binds to the single stranded template DNA and is digested by the 5′nuclease activity of AmpliTaq-Gold DNA polymerase. Digestion of the probe releases the fluorescent reporter dye (either FAM or VIC) from the quencher dye.

Determination of erythrocyte MnSOD enzyme activity [21]

MnSOD catalytic activity was determined with RANSOD kit (Randox Labs, Crumlin, UK) according to the manufacturer's protocol. In brief, SOD catalytic activity was measured by utilizing tetrazolium salt, which produces red formazan dye upon reduction with a superoxide anion ($O_2^{-\bullet}$) produced by xanthine and xanthine oxidase. MnSOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of tetrazolium salt. The test was performed on spectrophotometer BTA 350 (Biosystems S.A., Barcelona, Spain) by recording the increase in absorbance at 505 nm at 37 °C against air blank. Percentage inhibition of sample was used to obtain units of SOD from standard curve. MnSOD activity was expressed as Unit/ml.

Statistical analysis

The SPSS computer software, version 10.0 (Chicago, IL, USA) was used for data analysis.

Quantitative data were presented as mean ± SD for normally distributed data. For comparison of the groups' means, Student's *t*-test and ANOVA were used followed by Post Hoc test. Correlation between quantitative variables was done using Pearson's correlation coefficient (*r*). Qualitative data were expressed as frequency and percentage. Associations between categorical and dichotomous variables were tested using Pearson's chi-squared test or Fisher's exact test. The odds ratio (ORs) and corresponding 95% confidence intervals (CIs) were estimated. All tests were two tailed and considered significant at $P < 0.05$.

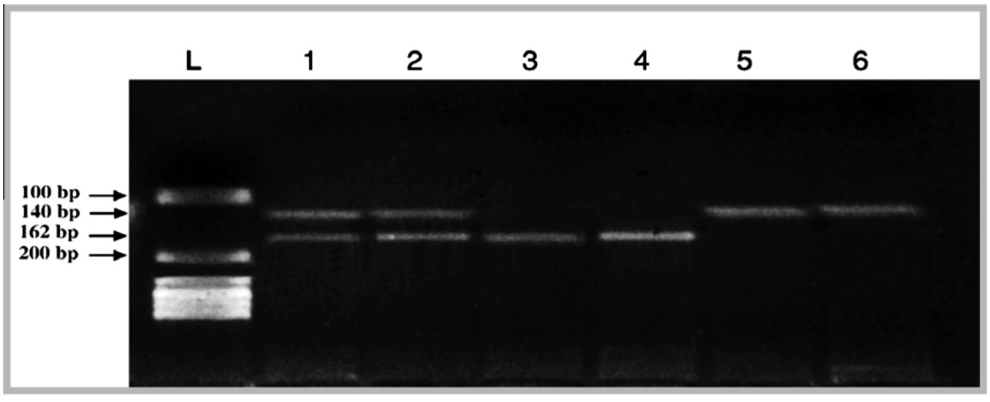

Figure 1 mEH exon 3 (Tyr 113 His) identified bands after transillumination by UV transilluminator: (L) DNA 100 bp ladder, lanes 1 and 2: heterozygous Tyr/His, lanes 3 and 4: homozygous mutant His/His, lanes 5 and 6: wild genotype Tyr/Tyr. (N.B.: The 22 bp band was too small to be detected).

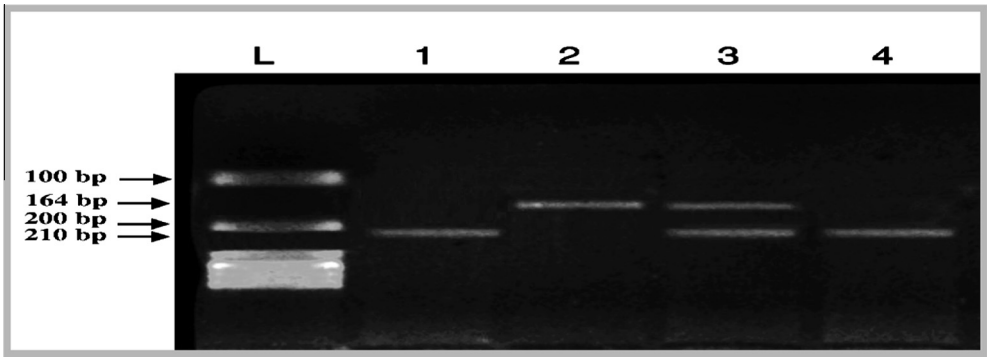

Figure 2 mEPHX exon 4 (His 139 Arg) identified bands after transillumination by UV transilluminator: (L): DNA ladder (100 bp), lanes 1 and 4: wild genotype (His/His), lane 2: homozygous mutant Arg /Arg, lane 3: heterozygous genotype (His/Arg). (N.B.: The 46 bp band was too small to be detected).

Results

The age, smoking index, enzyme genotype distribution and activity are presented in Table 1. mEH gene and exon 3 polymorphism, showed a significant difference in the genotype and allele distribution among the three groups. Homozygous mutant His/His genotype was absent among the passive smokers and presented in 16% of lung cancer group. While homozygous wild genotype Tyr/Tyr showed the highest frequency (90%) among the passive smoker group and was lowest among lung cancer group (56%) (P = 0.002). However exon 4 polymorphism of mEH gene and MnSOD Val16Ala showed

Table 1 Principle characteristics, antioxidant enzymes genotypes frequency and activity levels among the studied groups.

Gene	Lung cancer (n = 50)	Chronic smokers (n = 50)	Passive smokers (n = 50)	P-value
Age (years)	51 ± 11	47 ± 9	45 ± 10	
Smoking index (Pack/year)	29.5 ± 22.4	35 ± 16.4	–	–
mEH (exon 3) genotype				
Tyr/Tyr Frequency (%)	28 (56%)	33 (66%)	45 (90%)	
Tyr/His	14 (28%)	13 (26%)	5 (10%)	0.002
His/His		4 (8%)	0 (0%)	
Allele	8 (16%)			
Tyr	70 (70%)	79 (79%)	95 (95%)	<0.001
His	30 (30%)	21 (21%)	5 (5%)	
mEH (exon 4) genotype				
His/Hi Frequency (%)	36 (72%)	41 (82%)	43 (86%)	
His/Arg	8 (16%)	6 (12%)	5 (10%)	0.441
Arg/Arg	6 (12%)	3 (6%)	2 (4%)	
Allele				
His	80 (80%)	88 (88%)	91 (91%)	0.065
Arg	20 (20%)	12(12%)	9 (9%)	
MnSOD (Val 16Ala) genotype				
Val/Val Frequency (%)	17 (34%)	23 (46%)	21 (42%)	
Val/Ala	27 (54%)	19 (38%)	25 (50%)	0.446
Ala/Ala	6 (12%)	8 (16%)	4 (8%)	
Allele				
Val	61 (61%)	65 (65%)	67 (67%)	0.666
Ala	39 (39%)	35 (35%)	33 (33%)	
Predicted mEH activity	a	b	c	
Normal n (%)	19 (38%)	29 (58%)	38 (76%)	
Slow	12 (24%)	13 (26%)	5 (10%)	0.002
Very slow	7 (14%)	2 (4%)	0 (0%)	
Fast	12 (24%)	6 (12%)	7 (14%)	
MnSOD activity (mean ± SD) (U/ml)	2.3 ± 1.2 A	2.2 ± 1.1 A	2.9 ± 1.0 b	0.03

Groups bearing same initials are statistically indifferent at P value 0.05.

no significant difference in the genotype or allele distribution among groups (Table 1).

Predicted mEH enzyme activity among the studied groups showed that the prevalence of normal activity increased exponentially from lung cancer group to passive smokers, in contrast the frequencies of the very slow and the fast activities were highest among lung cancer group, this difference was significant ($P = 0.002$), while comparison between mean values of MnSOD activity level showed that the highest activity was recorded among the passive smokers and the lowest activity was recorded among lung cancer group, this difference was significant ($P = 0.03$) (Table 1).

A significant association between lung cancer risk with mEH exon 3 and 4 polymorphisms was revealed. Chronic and passive smokers carrying the mutant His allele of exon 3 polymorphism have 2.9 folds increased risk of developing lung cancer than carriers of the wild Tyr allele. Also those carrying the mutant Arg allele of exon 4 have 2.1 folds higher risk of lung cancer than carrier of the wild His allele. However no significant association was found between lung cancer risk and MnSOD genotypes or alleles (Table 2).

Correlation between cigarette smoking index (pack/years) and MnSOD enzyme activity (U/ml) showed statistically significant negative correlation ($r = -0.33$, $P < 0.001$) (Fig. 3).

Table 3 Relation between MnSOD enzyme activity and genotypes.

MnSOD Genotype	MnSOD activity (U/ml) (mean ± SD)	*P*-value
Val/Val ($n = 61$)	3.2 ± 0.8 a	
Val/Ala ($n = 71$)	2.3 ± 0.9 b	<0.001
Ala/Ala ($n = 18$)	0.7 ± 0.4 c	

Groups bearing same initials are statistically indifferent at *P* value 0.05.

Table 2 Odds Ratios (OR) for the (mEH) gene polymorphisms and MnSOD in lung cancer group versus chronic and passive smokers groups together.

Group	Lung cancer	Chronic and Passive smokers	OR (95%CI)	*P*-value
	Frequency (%)			
mEH (exon 3)				
Tyr/Tyr	28 (56%)	78 (78%)		
Tyr/His and His/His	22 (44%)	22 (22%)	2.1 (1.1–4.5)	0.046
Tyr	70 (70%)	174 (87%)		
His	30 (30%)	26 (13%)	2.9 (1.6–5.2)	<0.001
mEH (exon 4)				
His/His	36 (72%)	84 (84%)		
His/Arg and Arg/Arg	14 (28%)	16 (16%)	2.0 (1.1–4.6)	0.049
His	80 (80%)	179 (89.5%)		
Arg	20 (20%)	21 (10.5%)	2.1 (1.1–4.2)	0.024
MnSOD (Val 16 Ala)				
Val/Val	17 (34%)	44 (44%)		
Val/Ala and Ala/Ala	27 (54%)	44 (44%)	1.6 (0.7–3.7)	0.147
Val	61 (61%)	132 (66%)		
Ala	39 (39%)	68 (34%)	1.2 (0.8–2.0)	0.234

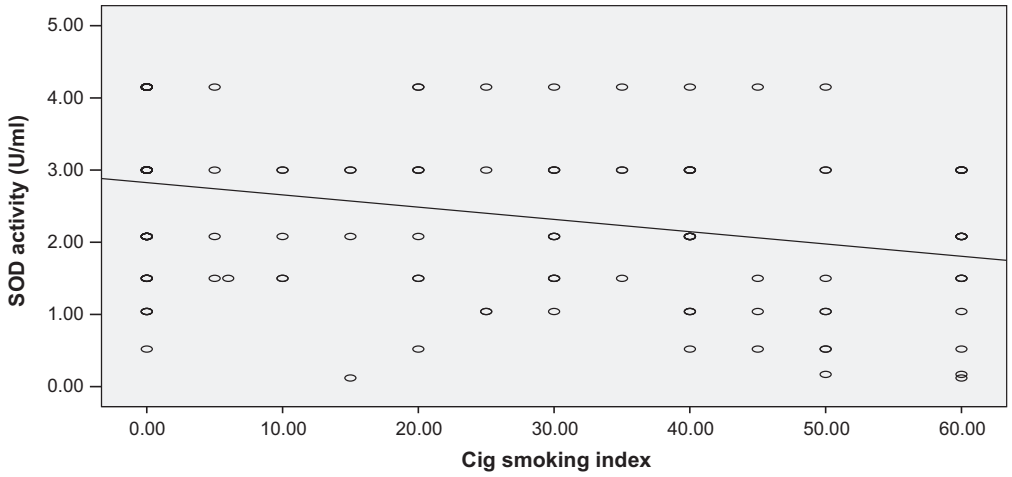

Figure 3 Scatter diagram showing correlation between cigarette smoking index and MnSOD enzyme activity (U/ml).

In a way to find an association between MnSOD activity and Val 16 Ala genotype, comparison of the mean values of MnSOD activity across the three MnSOD genotypes showed that the highest activity was recorded among the homozygous wild genotype (Val/Val) and the lowest activity was found among homozygous mutant genotype (Ala/Ala). This difference was significant ($P < 0.001$) (Table 3).

Discussion

Genetic differences in metabolic activation or detoxification of environmental carcinogen, like polycyclic aromatic hydrocarbons present in tobacco smoke, may partially explain host susceptibility to smoke induced cancers [21].

In this study, ETS exposed individuals (both active and passive smokers) who carry the mutant His allele of mEH exon 3 polymorphism have 2.9 folds increased risk of developing lung cancer **($P < 0.001$)**.

In agreement, Tilak et al. [22] in Indian population and Erkisi et al. [23] have also revealed that smokers with His/His genotype have significantly greater risk of lung cancer. On the other hand, exon 3 genotypes of mEH have been linked to a decreased lung cancer risk in several studies [24–26]. A meta-analysis by Wang et al. [4] found that pooled results of the Caucasians subgroup revealed that mEH exon 3 polymorphism is protective against lung cancer. However, in the Asians subgroup analysis, the pooled results showed an associated with increased risk of lung cancer. This suggested that there is an obvious race-specific effect.

In the present study, passive and active smokers who carried the Arg variant allele of mEH exon 4, polymorphism (fast allele) have 2.1 fold increased risk of developing lung cancer **($P = 0.024$)**. Similarly, Li et al. [26] and Liu et al. [27] showed that fast allele Arg was significantly associated with increased risk of lung cancer. These findings could be explained by the role of mEH enzyme in transforming epoxide intermediates into more reactive carcinogenic metabolites, which emphasizes that high mEH activity is not protective in smokers with a high cumulative dose of carcinogens derived from smoking [28].

In the present study, it was noticed that the fast and very slow predicted mEH activity showed the highest frequency in lung cancer group in comparison to chronic smokers and passive smokers **($P = 0.002$)**. It has been shown that cigarette smoking can significantly induce the activity of mEH [29]. Hydrocarbons such as benzo(α)pyrene are activated by mEH into reactive intermediates and higher mEH activity leads to higher concentrations of DNA adducts in the body compared with very slow activity of mEH [28], thus putative high mEH activity may have a harmful effect. Either low or high mEH metabolizers should be seriously considered for their ability to simultaneously decrease and increase the bioactivation of specific compounds [30]. The most commonly studied polymorphism of MnSOD is Val16Ala on mitochondrial target sequence [12]**.** However, the specific role of the Val16Ala SNP in cancer development was controversial.

In the current study, there was no significant difference in the MnSOD Val16Ala polymorphic genotypes or allele distribution among groups. Therefore, no significant association between MnSOD Val16Ala polymorphism and lung cancer susceptibility was detected **(*OR:* 1.2, $P = 0.234$)**.

In agreement, previous studies confirmed no association existing between MnSOD (Val16Ala) polymorphisms and risk of lung cancer [31,32]. However, since it was accepted that MnSOD constitutes a first-line defense against reactive oxygen species (ROS), it would be a premature conclusion to remark that this polymorphism has no role in lung cancer development. MnSOD Val16Ala polymorphism could not be linked to cancer risk alone. Rather, it has been proposed as low penetrance allele. However combination with other polymorphisms in linkage disequilibrium that up-regulate and down-regulate gene function, may be related to cancer risk [33]. Another point to be considered is that some polymorphisms might be associated with cancer risk for some ethnic communities only not for other ones.

In the current study, the MnSOD activity among lung cancer patients was significantly lower than in chronic or passive smokers **($P = 0.03$)**.

This was in accordance to many studies [16,31,34–36], in which the MnSOD activity in the lung cancer was lower than controls. The low activity of MnSOD in erythrocytes may be caused, at least in part, by oxidant damage to the protein MnSOD [16]. Another plausible explanation is due to increased utilization to scavenge free radicals [35] or sequestration by tumor cells [36]. These findings suggest that the lower levels of erythrocyte MnSOD activity in patients with lung cancer are likely the consequence of the disease process rather than a predisposing event in patients. This can explain the significant negative correlation that we found between cigarette smoking index (pack/year) and MnSOD enzyme activity (U/ml), thus, the extent of cigarette smoking could potentially affect MnSOD activity [11].

In agreement with the present study, Jain et al. [37] and Gavali et al. [38] have all found that erythrocyte MnSOD was significantly lower in cigarette smokers as compared to non-smokers.

Although mEH and Mn-SOD enzymes are considered to be in the first line of defense against oxidative stress, other enzymes such as catalase, glutathione peroxidase and glutathione reductase have major contribution in protecting cells from oxidative stress [39]. Hence, it can be expected that only a proper balance between the activities of these enzymes collectively can protect cells from detrimental effects of oxidative stress.

To sum up, this study indicated that mEH exon 3(Tyr113Hist) and exon 4 (Hist139Arg) polymorphisms that alter enzyme activity might contribute to lung cancer susceptibility among ETS exposed individuals through disturbed antioxidant balance. However, MnSOD Val16Ala polymorphism might not be associated with lung cancer risk, although MnSOD enzyme activity was significantly affected by smoking through its consumption in attacking ROS, leading to dysfunction of antioxidant defense mechanisms, yet it cannot be distinguished whether this decrease in activity is a risk factor for lung cancer or not. Governments should adopt and implement comprehensive smoke-free legislations in all public places and workplaces to protect all people from exposure to tobacco smoke.

Conflict of interest

The authors declare that they have no competing interests.

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